Investigations on the Toxic & Teratogenic Effects of GRAS Substances on the Developing Chick Embryo **Carob Bean Gum** No Date

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Investigations on the Toxic and Teratogenic Effects of GRAS Substances on the Developing Chick Embryo.[1]

Carob Bean Gum

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[1]Report of investigations conducted under Contract No. 72-343 with the Food and Drug Administration, PHS, DHEW.

General Protocol:

Ten test substances were supplied by the Food and Drug Administration for testing in the chick embryo. Details on the nature and source of these substances is shown in Table i. All substances were stored at room temperature in the dark until they were used, except that the propyl gallate and phosphated mono- and di-glycerides were kept under refrigeration. Most of the substances were dissolved in a suitable solvent or suspended in a suitable liquid for injection into fertile eggs. In one instance the substance was injected directly without a solvent or carrier. Specific information about solvents, solubility of the substances and problems peculiar to individual substances will be given under specific protocol for each substance tested.

Fertile eggs used in these investigations were from a specific pathogen free flock of Dekalb 161 egg production type chickens fed a breeder ration free of antibiotics or other drugs. Eggs were stored at 55° F and a relative humidity of 80 percent for 0 to 5 days prior to use. Eggs were allowed to reach room temperature, placed on plastic flats and subjected to ultraviolet irradiation for 30 minutes. The top of each egg was cleansed by a cotton swab saturated with 70 percent ethanol, a small hole was drilled over the air cell through the shell and the test substance was injected with the aid of a 0.25 ml. tuberculin syringe fitted with a suitable needle. All equipment and glassware used to handle the test substances or their solutions or suspensions were sterilized by auto claving and every attempt was made to avoid microbiological contamination of the eggs. Following injection the hole in each egg was sealed by a drop of flexible collodion and the eggs were set in or returned to the incubators. Jamesway Model 252 Incubator-Hatchers were used and maintained at 100° F dry bulb temperature and 86° F wet bulb temperature during the first 18 days of incubation. Eggs were turned automatically each 4 hours. Eggs were candled periodically to remove dead embryos and all embryos were examined for stage of development and obvious defects. After 18 days of incubation viable embryos were transferred to hatching baskets and hatching temperature was reduced to 96.5° F dry bulb reading and humidity was increased to a 90° F wet bulb reading. Upon hatching (22nd day) chicks were examined for abnormalities and samples were cleared and alizarin stained to examine them for skeletal defects. Other embryos (50 for each substance studied) were sacrificed and samples of liver, muscle, bursa, brain, eye, spleen, heart, pancreas, lung and kidney were taken and fixed in formalin. Later tissues were embeded in paraffin, cut, stained and mounted for histopathological examination. Each sample was done in duplicate and hence a total of 10,000 tissues were examined for lesions.

Preliminary range finding experiments were conducted to find the doses of the test substances that could be used in constructing dose response curves for toxicity as measured by embryonic mortality. In two cases, the test substance was non-toxic in the largest dose that could be accommodated by injection. Specific dose response experiments using 100 or more eggs per dose and 5 or more doses of the test substance were conducted at a minimum of 3 time intervals to obtain the toxicity data reported. Solvent or sham injected controls and untreated control groups of eggs were used with each experiment. In some cases, extra trials were conducted to provide embryos for examination at critical doses of the test substances in order to further evaluate teratogenic response and obtain additional data on the nature of embryonic defects.

Data obtained from the experiments (except that from the range finding studies) was transferred to data sheets provided (FDH form 2572, 2572a and 2572b) and submitted to FDA for statistical analysis. Nine types of data summaries including 2 statistical treatments of the data were provided by FDA on the data submitted. The results presented and interpretations made are largely based on these data summaries.

Table i

FDA Project Test Substances

Test Substance and Identification	Compound No.
1. Lactose, Edible Formost Dairies, Inc. Appleton, Wisc.	000063423
2. Propyl Gallate Lot 337	000121799
3. Sodium Ascorbate, U.S.P. FCC Lot No. 965102 Hoffmann-LaRoche Inc., Nutley, N. J. FDA 3167 73(C)	000134032
4. Sodium Erythorbate F.C.C. Lot No. 834072 FDA 3167 73(C) Hoffmann-LaRoche, Nutley, N. J.	977052064
5. Oil Nutmeg NF, East Indian Fritzsche Dodge & Olcott, Inc. 71-28 New York, N. Y.	MX 8008455
6. Zinc Sulfate - Rayon Lot # 2132R1 Virginia Chemicals, Inc. Portsmouth, Va.	Anhyd. 007733020 Monohyd. 007446197
7. Stannous Chloride, AR $2H_2O$ Mallinckrodt Chemical Works St. Louis, Mo.	007772998
8. Talc USP #141, Whittaker, Clark and Daniels, Inc.	010101390
9. Carob Bean Gum FDA 71-14	PM 9000402
10. Phosphated Mono- and Di-Glycerides Lot No. 126 Witco Chemical Organics Division New York, N. Y. EMCOL D70-30C	977051323

General Discussion and Comparisons:

A comparison of the relative toxicity of the ten compounds tested is shown in Table ii. When toxicity is evaluated by the air cell route of injection at 96 hrs. of incubation, which was the most sensitive for most of the substances tested, it may be seen that the test substances can be divided into 3 categories of toxicity. Substances highly toxic are zinc sulfate, propyl gallate and carob bean gum. Moderate toxicity was encountered with sodium ascorbate, sodium erythorbate, oil of nutmeg and stannous chloride. Those substances of low toxicity were lactose, talc and phosphated mono- and di-glyceride.

Most of the substances tested produced general embryo toxic response as ascites and/or edema except for lactose and talc at the doses tested. Some specific structural defects were noted and seemed to be related to certain substances as shown in Table ii.

Table ii

Comparison of Ten Substances Tested
for Toxicity and Teratology

Substance Tested	LC_{50} via air cell at 96 hrs.	Specific Abnormalities Noted
Lactose	very large	none
Iropyl Gallate	13 mgs./kg.	Ascites, edema, celosomia.
Sodium Ascorbate	100 mgs./kg.	Ascites, edema, celosomia, liver histopathology, head defects.
Sodium Erythorbate	84 mgs./kg.	Ascites, liver histopathology.
Oil of Nutmeg	240 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Zinc Sulfate	4 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Stannous Chloride	120 mgs./kg.	Ascites, edema, celosomia.
Talc	>200 mgs./kg.	none
Carob Bean Gum	23 mgs./kg.	Anophthalmia, phocomelia, micromelia, torticollis, celosomia.
Phosphated Mono- and Di-Glycerides	>3000 mgs./kg.	Ascites, anophthalmia, brachygnathia.

IX. CAROB BEAN GUM

Specific Protocol:

Carob bean gum was dissolved with continuous stirring and heating in 0.2 N hydrochloric acid. The resultant suspension-solution was diluted with sterile water to provide for various levels of administration. A water injected control was used since the unneutralized acid was highly toxic. Seven dose levels of carob bean gum were tested both at 0 and 96 hrs. of incubation and via both air cell and yolk routes of administration.

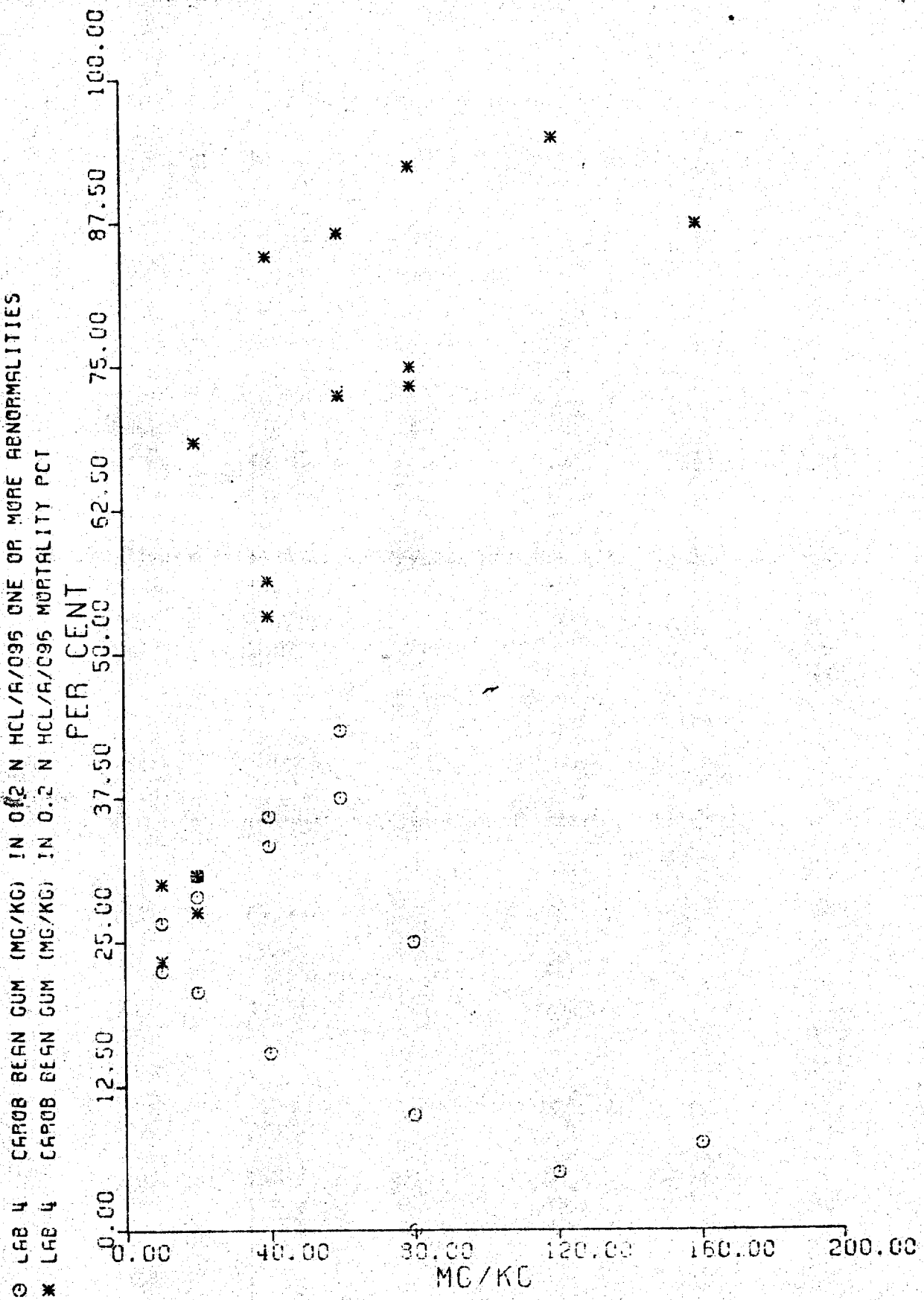
Results:

The data for carob bean gum is presented in Tables 33-36. Percent mortality was increased by a highly significant amount by one or more of the higher test doses and the regression of dose on mortality was significant or highly significant when carob bean gum was given via the air cell at either 0 or 96 hrs. When given via the yolk no significant effect of dose on percent mortality was observed. Percent abnormal chicks hatched and percent H-S-V-L abnormalities were increased by a highly significant amount at one or more doses of carob bean gum when given via the air cell at 96 hrs. Abnormalities were not significantly altered by the other 3 types of compund administration. Carob bean gum seemed to induce several specific embryonic abnormalities seen only at a very low frequency previously. When one examines the types of defects involved in the significant increase observed with air cell injection at 96 hrs. it is apparent that more head, limb and skeletal defects are observed along with increases in visceral defects noted for other substances. Anophthalmia; phocomelia; micromelia; torticollis, congenital; and celosomia seemed to increase in those groups showing considerable embryonic mortality. In addition to the abnormalities in hatched chicks, many of the dead embryos examined prior to hatching showed various head, skeletal, visceral and limb defects. In fact, carob bean gum was the only substance studied that seemed to greatly increase embryonic defects observed prior to hatching. This effect was again due to air cell administration of the substance.

Discussion:

The toxicity and teratogenicity of carob bean gum seems to be enhanced by dissolving it in 0.2 N hydrochloric acid. When the acid solution of this substance was neutralized it changed color and became partly insoluble. When this neutral suspension was tested, it failed to be as toxic or as teratogenic as the acid solution. Never-the-less, the acid solution was clearly toxic and teratogenic. The LC_{50} at 96 hrs. via the air cell was about 23 mgs./kg.

O LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/A/OOO ONE OR MORE ABNORMALITIES
* LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/A/OOO MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
0.00
40.00
80.00
120.00
160.00
200.00
MG/KG


⊖ LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/A/096 ONE OR MORE ABNORMALITIES
✱ LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/A/096 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
0.00
40.00
80.00
120.00
160.00
200.00
MG/KG

⊙ LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/Y/096 ONE OR MORE ABNORMALITIES
✱ LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/Y/096 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
0.00
40.00
80.00
120.00
160.00
200.00
MG/KG

LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/Y/000 ONE OR MORE ABNORMALITIES
LAB 4 CAROB BEAN GUM (MG/KG) IN 0.2 N HCL/Y/000 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
0.00
20.00
40.00
60.00
80.00
100.00
MG/KG ×10

Table 33

DATA SUMMARY

Carob Bean Gum in 0.2 N HCl
via Air Cell at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	448	6.47	5.80	1.33
Solvent	None	116	8.62	8.49	*
10.0	0.5	80	7.50	6.25	1.25
20.0	1.0	118	9.32	8.47	0.84
40.0	2.0	120	10.83	8.33	1.66
60.0	3.0	80	8.75	8.75	0
80.0	4.0	120	13.33	13.33	2.50[3]
120.0	6.0	40	17.5	5.00	0
160.0	8.0	40	30.0[1]	15.00[2]	0

[1] Difference from lowest test dose is highly significant

[2] NS

[3] NS

[4] Regression of dose on mortality is significant

LC_{30} = 626 mgs./kg.
LC_{50} = 5197 mgs./kg.
LC_{70} = 43,123 mgs./kg.
LC_{90} = 915,355 mgs./kg.

[5] $F\ (Cal) < F\ (.05)$

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 34

DATA SUMMARY

Carob Bean Gum in 0.2 N HCl
via Air Cell at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks[5] Hatched	Percent H-S-V-L Abnormalities
Control	None	448	6.47	5.80	1.33
Solvent	None	119	5.04	6.80	*
10.0	0.5	70	27.14	24.28	8.57
20.0	1.0	106	43.39	31.13	9.43
40.0	2.0	108	65.74	27.77	17.59
60.0	3.0	70	78.57	40.00[2]	45.71[3]
80.0	4.0	110	80.9	20.0	8.18
120.0	6.0	40	95.0[1]	5.0	5.0
160.0	8.0	40	87.5	7.5	0

[1] Difference from lowest test dose is highly significant

[2] Difference from test group showing least response is highly significant

[3] Same as 2

[4] Regression of dose on mortality is highly significant

LC_{30} = 12 mgs./kg.
LC_{50} = 23 mgs./kg.
LC_{70} = 47 mgs./kg.
LC_{90} = 130 mgs./kg.

[5] Slope is negative

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 35

DATA SUMMARY

Carob Bean Gum in 0.2 N HCl
via Yolk at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	448	6.47	5.80	1.33
Solvent	None	120	33.33	8.75	*
10.0	0.5	79	26.58	8.86	3.79
20.0	1.0	117	23.93	8.54	2.56
40.0	2.0	118	22.03	11.01	0.84
60.0	3.0	80	33.75	15.00	3.75[3]
80.0	4.0	118	25.42	11.01	1.69
120.0	6.0	39	28.20	15.38[2]	2.56
160.0	8.0	40	35.0[1]	0	0

[1] NS

[2] NS

[3] NS

[4] F (Cal) < F (.05)

[5] F (Cal) < F (.05)

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 36

DATA SUMMARY

Carob Bean Gum in 0.2 N HCl
via Yolk at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	448	6.47	5.80	1.33
Solvent	None	109	9.17	7.07	*
10.0	0.5	69	4.34	4.34	0
20.0	1.0	110	15.45[1]	8.18	1.81
40.0	2.0	107	8.41	12.14	1.86
60.0	3.0	68	10.29	5.88	2.94
80.0	4.0	110	10.90	10.00	0.90
120.0	6.0	40	15.00	15.00	0
160.0	8.0	40	7.50	17.50[2]	7.50[3]

1 NS
2 NS
3 NS
4 F (Cal) < F (.05)
5 F (Cal) < F (.05)